

AP3 Rec'd PFI/PTO 12 JUN 2009

IN THE SPECIFICATION:

Please amend the specification as follows:

On page 1, after the Title, please insert the following

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a national stage application of International Application PCT/JP2004/019196 filed December 22, 2004, which claims benefit of Japanese Patent Application No. 2003000425673 filed December 22, 2003, which are incorporated herein by reference in their entireties.

On page 6, please delete the paragraph at lines 7 through 18, and insert therefor the following paragraph:

As described earlier, many polyunsaturated fatty acid biosynthetic genes are cloned from various species of organisms. However, there is only a few reports in which polyunsaturated fatty acids having 20 or more carbon atoms with a degree of unsaturation 4 or greater, such as arachidonic acid and EPA, were produced in plants. As an example, it has been reported that *Phaeodactylum tricornutum*-derived $\Delta 6$ desaturase gene and $\Delta 5$ desaturase gene, and a *Physcomitrella patens*-derived $\Delta 6$ chain elongase gene were expressed in *Linum usitatissimum* to produce arachidonic acid and EPA. However, this is not described in detail (see Non-Patent Publication 24).

On page 10, please delete the paragraph beginning at line 20 through page 11, line 3, and insert therefor the following paragraph:

(7) A gene that encodes a *Marchantiales*-derived protein having a $\Delta 6$ chain elongating activity, and that (a) consists of a nucleotide sequence of from the 194th to 1066th nucleotides of SEQ ID NO: [[1]] 3, or (b) hybridizes under stringent conditions with a DNA

nucleotide sequence of from the 194th to 1066th nucleotides, or its complementary sequence, of SEQ ID NO: [[1]] 3.

On page 16, please delete the paragraph at lines 5 through 19, and insert therefor the following paragraph:

Previous reports suggest that the $\Delta 6$ desaturase, $\Delta 6$ chain elongase, and $\Delta 5$ desaturase are involved in both n-6 pathway and n-3 pathway. Specifically, the $\Delta 6$ desaturase in the n-6 pathway converts linoleic acid (~~18:2 Δ ^{9,12}~~ 18:2 Δ ^{9,12}, containing 18 carbon atoms, and two double bonds at positions 9 and 12 (the same notation will be used below)) into γ -linolenic acid (GLA; ~~18:3 Δ ^{6,9,12}~~ 18:3 Δ ^{6,9,12}). In the n-3 pathway, the $\Delta 6$ desaturase converts ~~a-linolenic~~ α -linolenic acid (ALA; ~~18:3 Δ ^{9,12,15}~~ 18:3 Δ ^{9,12,15}) into stearidonic acid (STA; ~~18:4 Δ ^{6,9,12,15}~~ 18:4 Δ ^{6,9,12,15}). The $\Delta 6$ chain elongase in the n-6 pathway converts GLA into dihomo- γ -linolenic acid (DGLA; 20:3 Δ ^{8,11,14}), and in the n-3 pathway converts STA into eicosatetraenoic acid (ETA; 20:4 Δ ^{8,11,14,17}). The $\Delta 5$ desaturase in the n-6 pathway converts DGLA into arachidonic acid (20:4 Δ ^{5,8,11,14}), and in the n-3 pathway converts ETA into eicosapentaenoic acid (EPA; 20:5 Δ ^{5,8,11,14,17})

On page 48, please delete the paragraph at line 23 through page 49, line 8, and insert therefor the following paragraph:

From the cDNA, clones c1 and c2 of different lengths (c1: 2,427 bp; c2: 2,285 bp) were isolated as candidates for the *Marchantia polymorpha*-derived $\Delta 5$ desaturase gene. By comparing the nucleotide sequences of the clones c1 and c2, it was found that alternative splicing had occurred in a 5' non-coding region. The alternative splicing did not change the reading frame, and both clones c1 and c2 coded for 484 amino acids (SEQ ID NO: 6). The clone c1 of 2,427 bp was used as a MpDES5 gene (SEQ ID NO: 5) in the following Examples.

On page 49, please delete the paragraph at lines 16-17, and insert therefor the following paragraph:

[Example 4: Functional analysis using methylotrophic yeast (~~*Pichia*~~ *Pichia pastoris*)]

On page 52, please delete the paragraph at line 12 through page 53, line 3, and insert therefor the following paragraph:

In an MpDES6 gene-expressing transformant, the products of the $\Delta 6$ desaturase reaction, ~~g-linolenic~~ γ -linolenic acid and stearidonic acid, were contained in 7.4% and 0.7%, respectively, with respect to the total fatty acids. In a pPICZA vector-introduced yeast used as a control, γ -linolenic acid and stearidonic acid were not detected. Thus, it was shown that the MpDES6 encoded the $\Delta 6$ desaturase.

In an MpELO1 gene-expressing transformant, 14.1% of the total fatty acids was dihomog-linolenic acid when γ -linolenic acid was added. On the other hand, 1.5% of the total fatty acids was eicosatetraenoic acid when stearidonic acid was added. In a pPICZA vector-introduced yeast used as a control, ~~dihomo-g-linolenic~~ dihomog-linolenic acid or eicosatetraenoic acid was not detected. Thus, it was shown that the MpELO1 encoded the $\Delta 6$ chain elongase.

On page 55, please delete the paragraph at line 5 through page 56, line 10, and insert therefor the following paragraph:

Using the transformants that have incorporated two or three kinds of genes, an experiment was conducted to reconstruct the arachidonic acid/EPA biosynthesis system of *Marchantia polymorpha*. First, using the transformants that have incorporated the two types of genes (MpDES6 and MpELO1), an MpDES6 ~~protein~~ gene and an MpELO1 ~~protein~~ gene were co-expressed in the methylotrophic yeasts. As a result, ~~g-linolenic~~ γ -linolenic acid and stearidonic acid, which are the products of $\Delta 6$ desaturation, were contained in 2.9% and 0.4%, respectively, with respect to the total fatty acids, whereas ~~dihomo-g-linolenic~~ dihomog-linolenic acid and eicosatetraenoic acid, which are produced by the chain elongation of the

~~g-linolenic~~ γ-linolenic acid and stearidonic acid, respectively, were contained in 2.8% and 0.2%, respectively, with respect to the total fatty acids. In the controls, these fatty acids were not detected. In the transformants that have incorporated three types of genes (MpDES6, MpELO1, and MpDES5), production of arachidonic acid (0.1% in the total fatty acids) and eicosapentaenoic acid (EPA, 0.03% in the total fatty acids) was confirmed, in addition to the ~~g-linolenic~~ γ-linolenic acid, stearidonic acid, dihomo-γ-linolenic acid, and eicosatetraenoic acid, which were contained in 2.8%, 0.5%, 1.5%, and 0.1%, respectively, with respect to the total fatty acids. In the controls, these fatty acids were not detected. This result showed that reconstruction of polyunsaturated fatty acid biosynthesis system is indeed possible in organisms other than *Marchantia polymorpha*, by expressing *Marchantia polymorpha*-derived Δ6 desaturase gene, Δ6 chain elongase gene, and Δ5 desaturase gene therein.

On page 62, please delete the paragraph at line 16 through page 63, line 3, and insert therefor the following paragraph:

This Example confirmed that the foregoing *Marchantia polymorpha*-derived unsaturated fatty acid synthetase genes, i.e. the MpDES6 gene, MpDES5 gene, and ~~MpELO~~ MpELO1 gene were indeed well functional in plants.

More specifically, by introducing the MpDES6 gene, MpDES5 gene, and ~~MpELO~~ MpELO1 gene into tobacco, production of arachidonic acid and other fatty acids were confirmed. For comparison, a tobacco was prepared into which filamentous fungus (*M. alpina*)-derived Δ6 desaturase gene (MaDES6), Δ5 desaturase gene (MaDES5), and Δ6 fatty-acid-chain elongase (MaELO) were introduced.

On page 66, please delete the paragraph at lines 8-21, and insert therefor the following paragraph:

In addition, the pUC19 was digested with HindIII and ligated to a PacI linker. By further digesting it with EcoRI followed by ligation to a FseI linker, pUCPF was obtained as a subcloning vector. Further, the pUC19 was digested with HindIII and ligated to a SgfI linker. By further digesting it with EcoRI followed by ligation to an AscI linker, pUCSA was

obtained. A vector in which EI235S was inserted into the HindIII-XbaI site of pUCSAPF, and in which a mannopin synthetase (mas) gene terminator was inserted into the SacI-EcoRI site of pUCSAPF was digested with XbaI and SacI and blunted to obtain pSPB2353A. To a blunt end of pSPB2353A, a DNA fragment containing the ~~MaDES6~~ MpDES6 gene which was excised from the p35S-MpDES6 with XbaI and blunted was ligated. As a result, pSPB2353 was obtained.

On page 67, please delete the paragraph at lines 12-21, and insert therefor the following paragraph:

PCR was run using a highly accurate KOD-plus-DNA polymerase (Toyobo) as an enzyme. The reaction was carried out at a maintained temperature of 94°C for two minutes, followed by 25 cycles of reaction at 94°C for 15 seconds and at 68°C for 1 to 3 minutes. An ~~MpELO~~ MpELO1 DNA fragment so prepared was digested with XbaI and SacI, and was ligated to the pSPB2355A to obtain pSPB2355. Further, a DNA fragment obtained by the digestion of pSPB2355 with SgfI and AscI was ligated to pSPB2353 digested with SgfI and AscI. As a result, pSPB2361 was obtained.

On page 68, please delete the paragraph at line 16 through page 69, line 3, and insert the following paragraph:

A DNA fragment obtained by the digestion of pBINSAPF with PacI and FseI was ligated to a DNA fragment containing the MpDES5 gene which was excised from pSPB2352 with PacI and FseI. As a result, pSPB2368A was obtained. Further, pSPB2368A was digested with SgfI and PacI and ligated to a DNA fragment containing the MpDES6 and ~~MpELO~~ MpELO1 genes which were excised from pSPB2361 with SgfI and PacI. As a result, pSPB2368 was obtained. The MpDES6 gene, MpDES5 gene, and ~~MpELO~~ MpELO1 gene were transcribed in the same direction on the plasmid pSPB2368, and were controlled by the same constitutive promoter.

On page 72, please delete the paragraph at lines 17-23, and insert therefor the following paragraph:

In the present Example, by introducing each gene of *Marchantia polymorpha*-derived $\Delta 6$ desaturase, $\Delta 5$ desaturase, and chain elongase into tobacco, 10% or greater arachidonic acid was accumulated in a tobacco leaf. This result suggests that the pSPB2368-transformed tobacco in the present Example is capable of more efficiently synthesizing polyunsaturated fatty acids, as compared with the foregoing report.